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PRINCIPAL INVESTIGATOR: Anindya Dutta, M.D.,Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

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Anindya Dutta, M.D.,Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, Massachusetts 02115

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13. ABSTRACT *(Maximum 200 words)*

Our aim is to dissect the interactions of p21 (a downstream effector of the tumor suppressor protein p53) with its two target, cyclin-cdks and PCNA. In year 1 we have established conditions for quantitatively analyzing the inhibition of cyclin-cdk by p21. This will enable us to measure the exact contribution of the interaction between the cyclin-binding Cy motif of p21 with a docking site on the cyclin in the inhibition of kinase activity. The conditions obtained have already demonstrated qualitatively the importance of the Cy motif in kinase inhibition. Different methods of site directed mutagenesis are being tried to determine the optimal method by which to generate a library of mutants in the Cy motif of p21. In year 2 this library of mutants will enable us to quantitatively determine exactly what constitutes a functional Cy motif. In order to dissect the interaction of p21 with PCNA we now have mutant forms of PCNA that fail to interact with p21 or with the exonuclease Fen1 without disrupting the trimeric structure of PCNA. This is the first step towards analyzing how the p21-PCNA interaction impinges on DNA replication and repair in cancer cells.

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INTRODUCTION:

Importance of p53 function in breast cancer: As discussed extensively in the original grant proposal, the p53 protein is an important tumor suppresser which is inactivated by mutation in up to 50% of breast cancers. In multiple studies, mutation of the p53 gene in a breast cancer is associated with shorter disease free interval and decreased overall survival, independent of the presence of axillary node metastases. In a small group of familial breast cancer patients (with Li Fraumeni syndrome), inactivating germ-line mutations are seen in the p53 gene, indicating the importance of normal p53 in preventing the appearance and progression of breast cancers.

p53 and p21: Both the germinal and the somatic mutations of p53 which are seen in cancers appear to be "loss of function" mutations. p53 is believed to suppress cell growth by the transcriptional induction of genes that negatively regulate cell growth. One such gene, discovered is the p21 gene (1).

p21 belongs to one class of cdk inhibitors which are related to each other in their primary sequence and their substrate specificity. These associate with and inhibit almost all cyclin-cdk pairs, and the three members of this class are p21, p27 and p57.

Domain structure of p21: Beside inhibiting cyclin-cdk kinases, the p21 protein directly interacts with and inhibits an essential DNA replication factor, proliferating cell nuclear antigen (PCNA) (2-4). The N terminal domain of p21 (p21N) interacts with the cdk2 protein and inhibits cyclin-cdk kinase activity, while the C terminal domain (p21C) interacts with and inhibits PCNA, the ring shaped sliding clamp that tethers DNA polymerases delta and epsilon to the replicating strand.

The cyclin binding motif in p21: Further dissection of the cdk inhibitory domain present in p21N demonstrated that a cyclin binding motif on p21 allows it to directly bind to cyclins (5). The cyclin binding sequence, ACRRLFGPV, is highly conserved in the other cdk inhibitors, p27 and p57, and is also conserved in substrates and activators of cdks. A twelve amino acid peptide containing this motif is sufficient to interact with cyclins. Together with our biochemical data, the crystallographic structure of cyclin A-cdk2 complexed with p27 (6), suggests that the Cy motif- cyclin interaction serves as a docking interaction essential for the complete interaction between cdk inhibitors and cyclin-cdk. In this project we shall determine how the cyclin binding site of p21 contributes to inhibition of cyclin-cdk kinase, and the implications of this discovery for designing chemicals that mimic p21 action by binding cyclin or cdk2.

The effect of p21 on the function of PCNA. PCNA inhibition by p21 is also important for growth control (7, 8). We have recently shown that in serum-starved diploid fibroblasts, the introduction of the PCNA inhibitory portion of p21 (without the cdk kinase inhibitory domain) is also sufficient to inhibit cell growth (8). Hence, in the second portion of this proposal we shall study the mechanism by which p21 affects the function of PCNA.

p21 disrupts the association of PCNA with the 5'-3' exonuclease Fen1. Prokaryotic DNA replication polymerases possess three activities: a 5'-3' DNA polymerase, a 3'-5' exonuclease (the proof-reading activity) and a 5'-3' exonuclease. None of the DNA polymerases identified in human cells possess the last activity. A 5'-3' exonuclease is however essential for complete DNA replication, and has been purified by virtue of its requirement in the SV40 based DNA replication reaction (9). In the absence of this special exonuclease the ribonucleotide-deoxyribonucleotide bond at the 5' ends of Okazaki fragments is not removed and consequently the Okazaki fragments are not ligated. This exonuclease has been independently identified as a flap endonuclease (Fen1) required for DNA recombination (10, 11) and as a gene essential for (a) cell-cycle progression and (b)

protection from radiation induced DNA damage in yeast S. cerevisiae (YKL510 or RAD27 or ERC1) and S. pombe (rad2) (12-14). Mutation of this gene in S. cerevisiae also produces an instability of direct repeats much like the instability seen in several human cancers (15).

We reported that Fen1/Rad2 directly associates with PCNA and that p21 disrupts the association of Fen1 with PCNA (16). This newly discovered activity of p21 is likely to be important for the propagation of DNA mutations following DNA replication, because unlinking Fen1 from PCNA by p21 may promote the same type of genomic instability as seen when RAD27 is mutated in S. cerevisiae. In this proposal we shall seek derivatives of PCNA which have uncoupled the PCNA-pol delta and PCNA-Fen1 interactions. These studies will in addition illuminate whether the complete action of p21 on the DNA replication apparatus requires that both these interactions of PCNA be breached. The results will determine whether uncoupling of the polymerase from the exonuclease by p21 contributes to the genomic instability seen in several breast cancers and also indicate whether the PCNA-Fen1 complex could on its own be a suitable target for chemotherapy.

BODY

Original Statement of work

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-12: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.

Task 2: Months 12-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.

Task 2: Months 24-36: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what sequence feature is essential for binding to cyclins.

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.

The underlined tasks are being done now. We have slightly re-ordered our priorities in Objective 1 because obtaining the mutant forms of the cyclin-binding motif (originally planned for months 24-36) will significantly improve our measurements of binding affinity of cyclin-binding sites for cyclins and the contribution of these sites to the association of p21 with cyclin-cdks (Task 1) . Hence from this objective, Task 2 is being attempted now as Task 1 and the original Task 1 has been moved back. This is reflected in the revised Statement of Work below:

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-24: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what sequence feature is essential for binding to cyclins.

Task 1: Months 1-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.

Task 2: Months 24-36: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.

Task 3: Months 24-48: Creation and testing of mutations of p21 with different affinities for cyclins D1 and E.

Task 4: Months 24-48: Creation and testing of versions of p21 with variation of the distance between the cyclin-binding and the cdk2 binding sites (in cis).

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.

Task 6: Months 12-24: Creation of p21 derivatives and determination of their IC90 on the processivity of PCNA-polymerase delta.

Task 7: Months 24-36: Creation and testing of PCNA mutants that have lost the interaction with Fen1 but can still stimulate polymerase delta

Task 8: Months 36-48: Testing effect of adding Fen1 to replication reactions or over-expressing Fen1 in MCF-7 cells in culture.

METHODS.

Inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin-binding site.

The substrate used was GST-CDC25A produced in bacteria and purified on glutathione agarose beads.. The enzyme (cyclin E-cdk2) was prepared by co-infecting SF9 insect cells with two baculoviruses expressing GST-cyclin E and cdk2. Active kinase complex is purified on glutathione agarose beads. Both substrate and enzyme were eluted with glutathione.

To determine initial velocity conditions for the kinase reaction, 4 ng GST-cyclin E/cdk2 was incubated with 2 or 0.2 μg of GST-CDC25A in 25 μl reaction buffer containing 50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 mM DTT, 50 μM ATP containing 5 μCi gamma 32P-ATP (sp. activity 3200 cpm/pmol). Reaction was allowed to proceed for indicated periods of time at 37 deg. c and stopped by boiling in Laemmli sample buffer. Products were separated by gel electrophoresis, GST-CDC25A visualized by autoradiography, and radioactivity incorporated into the band counted by liquid scintillation counting.

To determine conditions for inhibition by p21, bacterially produced GST-p21 was purified by glutathione agarose affinity chromatography and titrated into the reaction (from 0.1 to 1000 ng). In addition p21 derivatives with deletions in the Cy motif (Δ17-24) and K motif (Δ53-58) and 12 mer peptides containing the Cy motif (PS100) and with a mutation in the Cy motif (PS101) were tested for their inhibition.

Mutagenesis of the Cy motif of p21.

The following strategies were tried in parallel.

1) A PCR-based strategy. Diverging oligonucleotides were made that annealed to the Cy motif region of p21 but incorporated specific mutations. PCR with these oligonucleotides and others that anneal to the ends of p21N cDNA generated fragments of 90 and 200 nucleotides which were then annealed to each other (through their overlap at the Cy motif region and re-PCR-ed with the end-specific oligonucleotides). The goal was to produce a 270 base-pair long fragment that would contain the mutant Cy motif which would then be cloned into the pGEX vector to produce GST-p21N with point-mutation in the Cy motif.

2) A cassette based strategy. PCR-based mutagenesis of GEX-p21N introduced a HindIII site at the N terminal end of the Cy motif (a silent mutation). Together with a naturally occurring BlpI site on the C terminal side of the Cy motif, this allowed us to excise the wild-type Cy motif. Synthetic oligonucleotides were annealed so that a mutant Cy motif was encoded by the cassette and cloned between the HindIII-BlpI sites of GEX-p21N.

3) The Kunkel method of mutagenesis. p21N was cloned into a vector (Blue-Script) that can produce single-stranded DNA. deoxy-Uridine containing single-stranded DNA template was prepared in CJ236 (*dut, ung*) bacteria. Annealing of mutant oligonucleotides (containing a point-mutation in the Cy motif) to this template followed by E. coli Klenow polymerase directed in vitro synthesis and transformation into wild-type (dut+,, ung+) bacteria is expected to select against the deoxy-Uridine containing single-stranded DNA template so that most of the colonies obtained will encode the mutant Cy motif.

Determination of the part of PCNA that interacts with p21 and Fen1.

A report appeared in the Literature (17) that indicated residues QLGI in the inter-domain connecting loop of PCNA was important for the association of PCNA with p21 and dispensable for forming a PCNA trimer. Plasmids encoding the mutant forms of PCNA described in that report were obtained from the authors. Bacterially produced GST-p21 and to GST-Fen1C (containing the C terminal portion of Fen1 which interacts with PCNA) were bound to glutathione agarose beads and their association with PCNA assayed as described by us (16). The wild type and mutant forms of PCNA were produced in two ways: (a) by in vitro-transcription translation in rabbit reticulocyte lysates and (ii) by expressing in E. coli. PCNA produced by in vitro transcription-translation was labeled with 35S methionine and visualized by fluorography, while the bacterially produced PCNA was visualized by immunoblotting with commercial anti-PCNA antibody.

RESULTS AND DISCUSSION

Inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin-binding site.

Fig. 1 indicates that with 2 μg of GST-CDC25A the kinase activity is linear beyond 10 minutes. Thus for the inhibition assays the kinase reaction was stopped at 10 minutes.

Fig. 2A shows that compared to wild type p21, p21Δ17-24 (deletion in one of the cyclin binding motif, Cy1) is almost 50 fold weaker in its inhibitory potency, while p21Δ53-58 (deletion in the cdk binding K motif) is about 8 fold weaker. This surprising result indicates that for certain substrates the Cy1 motif of p21 is even more important than the K or Cy2 motifs for inhibition of cyclin E-cdk2. In the future, we will examine whether the same holds for cyclin A-cdk2 (which might prefer the Cy2 motif near the C terminus of p21 over the Cy1 motif in the N terminal half). We will also examine the inhibitory reaction with a mutant substrate lacking a Cy motif, GST-CDC25A2 (18).

Collectively these experiments will indicate the relative importance of each Cy motifs (both on the inhibitor and on the substrate) for kinase inhibition. In addition these experiments will reveal whether there is specificity encoded in the Cy motifs (Cy1 or Cy2) with regard to which cyclin (A or E) is preferentially targeted.

Fig. 2B shows that PS100, the 12 amino acid peptide containing the Cy motif (ACRRLFGPVDSE), inhibits the activity of cyclin E-cdk2 with an IC50 of about 200 ng/25 μL (6 μM). In contrast the mutant peptide, PS101 (ACRRLKKPVDSE) is inactive up to 10000 ng (300 μM). In comparison an IC50 of 200 nM for PS100 was reported when cyclin E/cdk2 was used to phosphorylate GST-RbC (the C terminal portion of the retinoblastoma protein) (5). The 12 fold decrease in potency of PS100 on CDC25A could be accounted by the Cy motif on CDC25A (the substrate used here) having a higher affinity for cyclin E/cdk2 than the Cy motif on Rb. This question will be addressed by using peptides that encode Cy motifs from other substrates of cdks (Rb, CDC25A and CDC6/Cdc18).

Mutagenesis of the Cy motif of p21.

Of the three methods considered, we had initially focused on a PCR based strategy to change the first R (R1 to A) and the second R (R2 to A) of the Cy motif of GST-p21N. However, we failed to obtain the correct mutant by this method.

We then tried the cassette based mutagenesis strategy. This is the method that we hope to optimize because if successful, cassettes with degenerate oligonucleotides will provide an easy way to produce a library of mutants in the Cy motif of GST-p21N. Two types of ligations have been tried using the R1 to A and R2 to A mutagenic oligos for optimization. Unphosphorylated oligos have been ligated to un-phosphatased GEX-p21N plasmid cut open with HindIII and BlpI. For comparison, kinases oligo-cassette has been ligated to GEXp21N plasmid linearized by HindIII and BlpI and de-phosphorylated with shrimp alkaline phosphatase. Using the first ligation approach we obtained mutants at an unusually low frequency (2 out of 18), which is sub-optimal for making a library of mutants. Currently we are awaiting the results from the second ligation approach.

Determination of the part of PCNA that interacts with p21 and Fen1.

In vitro transcribed -translated PCNA (wild type and mutants) were bound to glutathione agarose beads coated with GST-p21 , GST-Fen1C and GST (negative control). No difference was seen in the binding of the mutants to p21 or Fen1 (not shown), a result that contradicts with the published report (17). We reasoned that non-radioactive wild-type PCNA subunits in the rabbit reticulocyte lysate may form heteromers with the radiolabeled mutants and facilitate their association with p21 or Fen1 giving a false positive result. Wild type and mutant PCNA were therefore synthesized in E. coli (which do not contain wild type eukaryotic PCNA), lysates containing the PCNA prepared, and the binding of PCNA to GST, GST-p21 and GST-Fen1C assayed. Fig. 3 shows that wild type PCNA and several of the mutants successfully bound to p21 and to Fen1. However, in agreement with published results, a mutation that changed the QLGI in the inter-domain loop of PCNA to AAAA, resulted in PCNA that failed to bind to p21 and Fen1.

Therefore we have at least one derivative of PCNA that has lost interaction with Fen1 and p21. PCNA with QLGI changed to AAAA has been purified to homogeneity over nickel-resin and Mono-Q columns (Fig. 4) preparatory to analyzing its effect on polymerase delta and its effect on genomic stability of direct repeats in an in vitro DNA replication reaction. In addition, finer mutations will now be made in and around the QLGI

region of PCNA to produce mutant forms of PCNA that selectively lose interaction with p21, Fen1 or polymerase delta. If such mutant forms of PCNA are found, over-expression of such proteins during in vitro or in vivo replication reactions will address the relative roles of these interactions in (a) maintenance of genomic stability and (b) the effect of p21 on DNA replication and genomic stability.

CONCLUSIONS

The cyclin-binding motif of p21 appears to be much more important than the cdk binding K motif for inhibiting the phosphorylation of substrates like CDC25A that contain exactly similar cyclin-binding Cy motifs. Future experiments will establish whether this is true by quantitative comparisons between substrates with different Cy motifs and inhibitors (p21 or synthetic peptides) with different Cy sequences. Preliminary experiments are still in progress to help select the optimal method of mutagenesis to make a library of mutant Cy motif in p21. The best method we have till now is successful at the rate of 2 out of 18 clones. If further improvement is not obtained by the other methods, we shall use this method to generate the mutants and test them. Mutant forms of PCNA have been generated that fail to interact with both p21 and Fen1. Finer mutations in PCNA over this region will allow us to distinguish the relative importance of PCNA-pol delta, PCNA-Fen1 and PCNA-p21 interactions for the inhibition of DNA replication and the maintenance of genomic stability

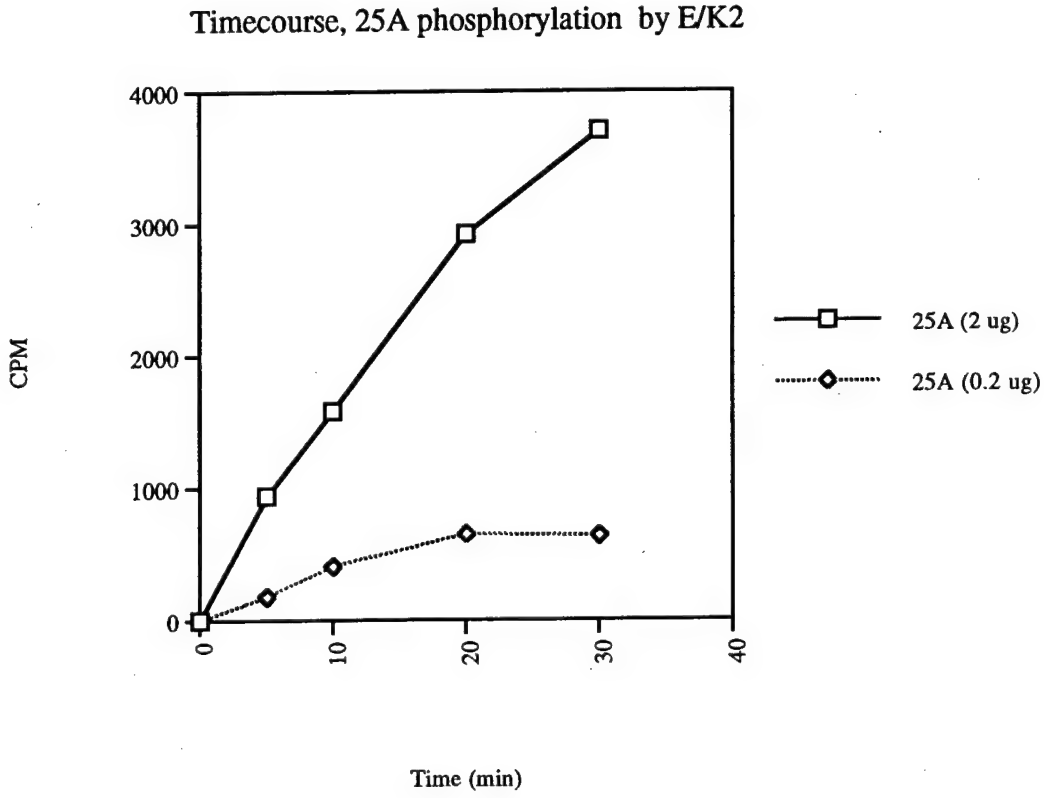
FIGURE LEGENDS

Fig. 1 Rate of phosphorylation of a substrate GST-CDC25A by cyclin E-cdk2 under conditions described in the text. Y-axis: cpm of 32P incorporated in the GST-CDC25A band. X-axis: Time of kinase reaction.

Fig. 2 Inhibition of cyclin E-cdk2. **(A)** Addition of indicated amounts of GST-p21 (squares), GST-p21 Δ17-24 (circles; deletion of cyclin E binding motif) and GST-p21 Δ53-58 (diamonds; deletion of cdk2 binding motif). The amount of 32P incorporated into GST-CDC25A at 10 minutes (initial velocity conditions) is expressed as percentage of 32P incorporated in the uninhibited reaction (100% kinase activity). **(B)** Addition of synthetic peptides PS100 (squares; wild type Cy1 motif) and PS101 (diamonds; mutant Cy motif). The rest is the same as in part A.

Fig. 3 QLGI PCNA fails to bind to Fen1 or to p21. PCNA bound to GST (GEX), GST-FEN1C (GEX-FEN1C) and GST-p21 (GEX-p21) was visualized by immunoblotting with anti-PCNA antibody (top). 10% of the PCNA input into the reactions is directly visualized by immunoblotting (bottom). **wt**: wild-type PCNA. **VDK**: Val188, Asp189 and Lys190 of PCNA changed to Ala. **QLGI**: Gln125, Leu126, Gly127 and Ile128 of PCNA changed to Ala. **SHV**: Ser43, His44 and Val45 of PCNA changed to Ala. **LAPK**: Leu251, Pro253 and Lys254 of PCNA changed to Ala.

Fig. 4 QLGI125 to AAAA form of PCNA purified to homogeneity and visualized by Coomasie Blue staining after SDS-polyacrylamide gel electrophoresis.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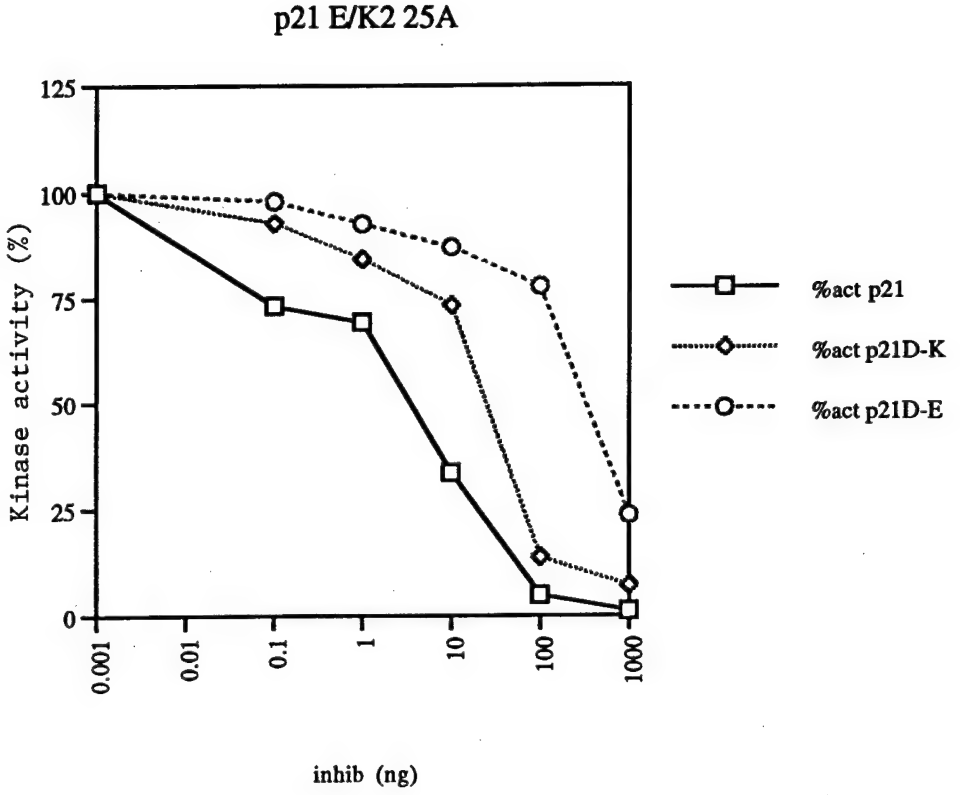
25A (2 ug)
25A (0.2 ug)

Fig. 1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50
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Kinase activity (%)
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inhib (ng)
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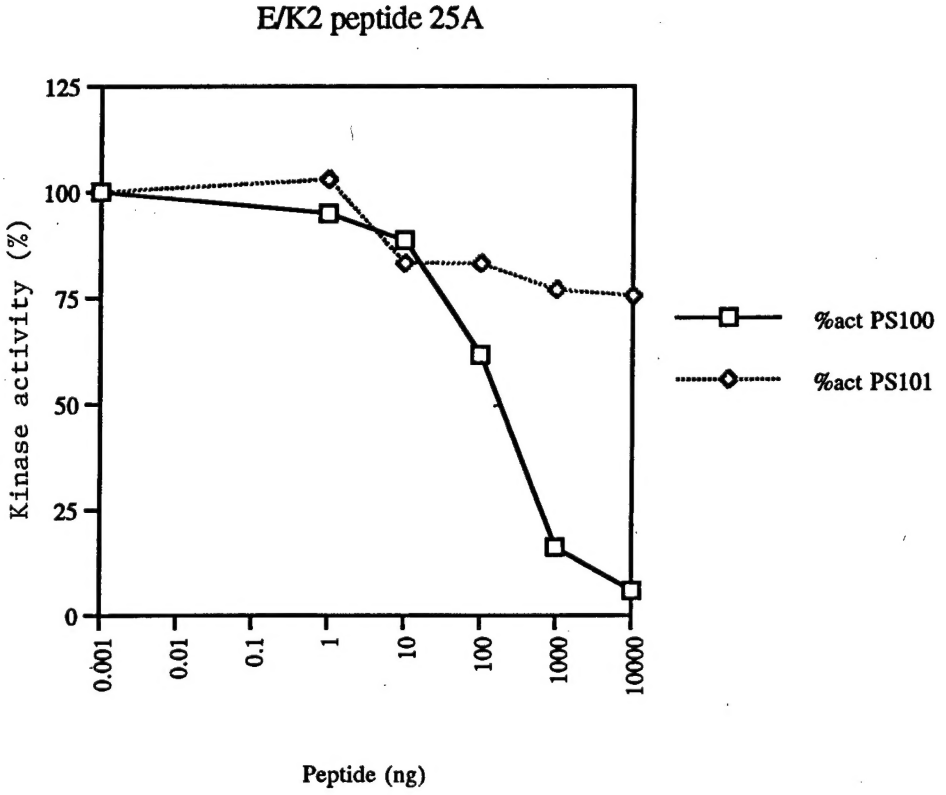
Fig. 2A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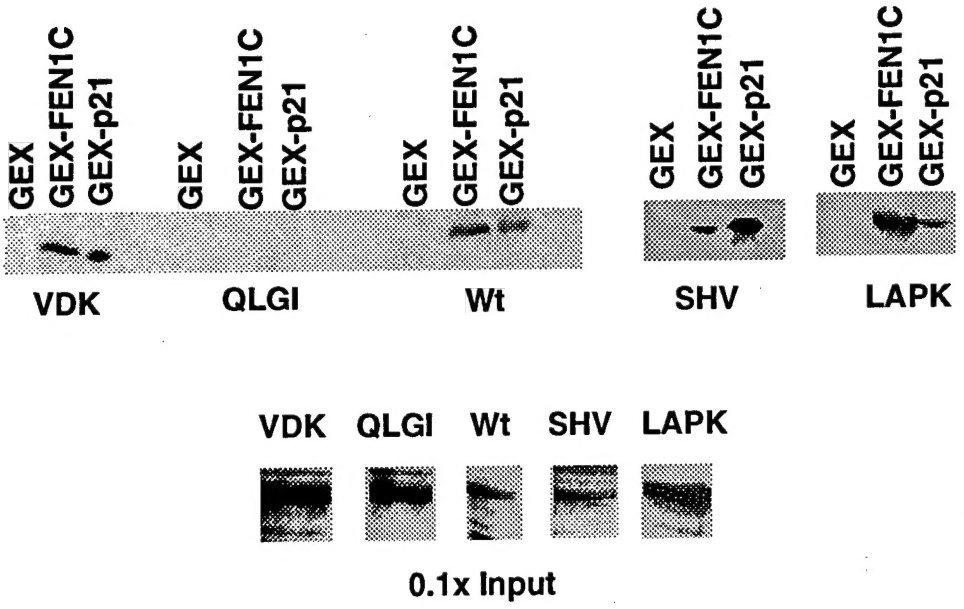
%act PS101

Fig. 2B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GEX-p21
VDK
QLGI
Wt
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SHV
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Fig. 3

Purification of PCNA Mutant QLGI

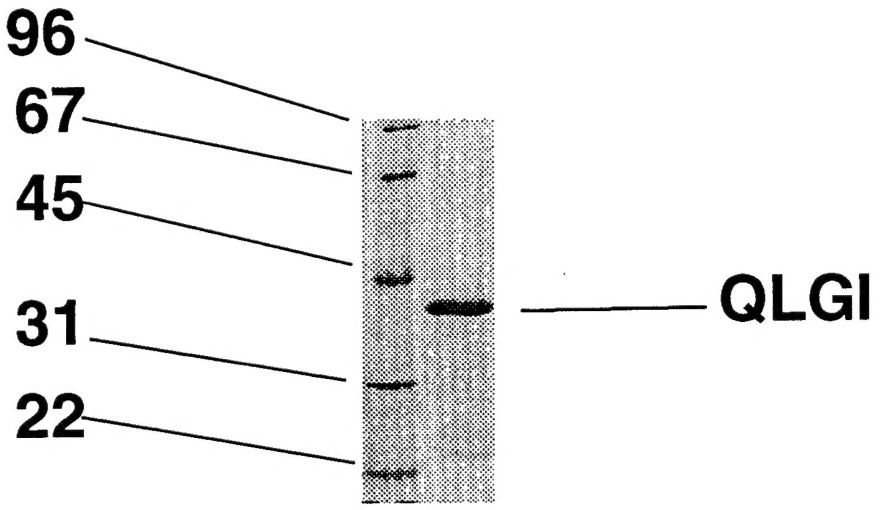

Fig. 4

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